Electronic Supplementary Information

Surface Modification: Activation and Deactivation of Osteogenic Differentiation Based on Detachable Growth Factor Protein

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Experimental Details

CVD polymerization

The PPX-ss-amine coating was prepared by using a home-built CVD system and was polymerized from the starting material (dimer) of 4-(2-amide-2'-amine-dithiobisethyl) [2.2]paracyclophane. The dimer was produced following procedures reported elsewhere.[1] During the CVD polymerization process, the dimeric 4-(2-amide-2'-amine-dithiobisethyl) [2.2]paracyclophane was sublimated at approximately 120-125 °C. The sublimated dimer was then transferred to a pyrolysis furnace in which the temperature was controlled at approximately 550-570 °C, and the dimer was transformed to become quinodimethanes (monomers). Subsequently, the radicals were further transferred into the deposition chamber and then polymerized onto a cooled substrate with a temperature of 25 °C to result in the PPX-ss-amine coating. The CVD system pressure was maintained at 75 mTorr throughout the CVD polymerization process, and a rate of approximately 0.5-1.0 Å/s was used for the deposition as monitored on the basis of a real-time quartz crystal microbalancing (QCM) device (STM-100/MF, Sycon Instruments, USA) that was installed on the deposition chamber.

Biomolecule immobilization and detachment

Recombinant human BMP-2 was obtained commercially (R&D Systems, USA) and was reconstituted as a stock solution at a concentration of 100 μg/mL in sterile 4 mM HCl and stored at -20 °C. The immobilization of BMP-2 on the PPX-ss-amine coating was performed by incubating the BMP-2 stock solution on PPX-ss-amine modified substrates with the addition of 2.5% glutaraldehyde (Sigma Aldrich, USA) in 50 mM carbonate buffer solution (pH 9.6) for 2 h at 37 °C. The reacted substrates were then washed three times with PBS containing Tween 20 (PBS-Tween 20, pH = 7.4, Sigma Aldrich, USA) and one time with pure PBS (pH=7.4, Sigma Aldrich) to remove the excess and unreacted BMP-2 and glutaraldehyde from the sample surface. The detachment of BMP-2 was performed by exposing the modified substrates to 10 mM glutathione (GSH, Sigma Aldrich) aqueous solution for 1 h at 37 °C followed by the same rinse process of PBS-Tween 20 and pure PBS to remove the cleaved molecules.

Characterization

Infrared reflection absorption spectroscopy (IRRAS) spectra were recorded using a 100 FT-IR spectrometer (PerkinElmer, USA) equipped with an advanced grazing angle specular reflectance accessory (AGA, PIKE Technologies, USA) and a liquid nitrogen-cooled MCT detector. The samples were mounted in a nitrogen-purged chamber, and the recorded spectra were corrected for any residual baseline drift. The dynamic binding analysis exploited an ADS QCM instrument (ANT Technologies, Taiwan), and the sensing element of the QCM instrument was an AT-cut piezoelectric quartz disc featuring a 9-MHz resonant frequency and a 0.1-cm^2 total sensing area. The quartz discs were coated with PPX-ss-amine via the CVD polymerization process and used for the immobilization/detachment of BMP-2. The changes in frequency before and after GSH treatment were monitored. Control experiments of immobilizing BMP-2 on the non-detachable PPX-amine were also conducted for comparison. Each experiment was conducted in triplicate.

Cell culture and flow cytometry

Bone marrow mesenchymal stem cells (BMMSCs) were isolated and purified by a reported method.[2] The immunophenotypes of the isolated BMMSCs were examined by flow cytometric assay. Cells were reacted in a dark room for 30 min at 4 °C according to the manufacturers' protocols with the following monoclonal antibodies: CD11b, CD29, CD31, CD34, CD45, CD86, and CD90. A FACS Calibur (BD Biosciences, USA) was used to analyze 2×10^5 BMMSCs, and the data were processed using the FCS Express software (Denovo Software, USA). All antibodies included isotype-matched control antibodies and were obtained commercially from eBiosciencem (USA) and BD Biosciences (USA). The resulting isolated BMMSCs were seeded on the modified culture substrates with an initial density of 5×10^3 cells/cm^2 in growth medium consisting of MEM alpha (Thermo Fisher Scientific, USA), 10% fetal bovine serum (FBS, Biological industries, Israel), and 1% antibiotic-antimycotic (Biological industries, Israel).

The growth medium was refreshed every 2-3 days. The viability of the cultured BMMSCs on the modified surfaces with subsequently induced GSH treatment was examined by adding GSH to the culture medium to a final concentration of 10 mM (pH was adjusted to 7.4 with 11.6 mg/ml $NaHCO_3$) for 1 h. Samples included the GSH treatment at day 0, day 4, day 8 and day 12. After replacing with fresh culture medium, the cell viability was determined using a Cell Counting Kit-8 (CCK-8, Beyotime Inst Biotech, China) according to the manufacturer's instructions, and the absorbance at wavelengths of 450 nm and 690 nm was detected by using a microplate reader (Bio-Rad, USA). Each experiment was conducted in triplicate. The morphology of the BMMSCs was observed and photographed under an inverse phase contrast microscope (Olympus IX71, Japan).

Differentiation and proliferation of BMMSCs

The differentiation capabilities of the isolated BMMSCs were verified. To induce adipogenesis, near-confluent monolayers of BMMSCs were cultured in MEM alpha supplemented with 10% FBS, 10 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, and 100 μM indomethacin for 21 days with medium changes every four days. Cells were fixed with 10% formaldehyde at room temperature for 10 min, and lipid droplets were stained using Oil Red O (Sigma Aldrich). To induce osteogenesis, confluent cells were incubated in MEM alpha supplemented with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerol phosphate and 50 μM ascorbic acid for 14 days with medium changes every four days. Bone matrix mineralization was evaluated using Alizarin red (Sigma Aldrich) staining for calcium deposition analysis. A pellet culture system was used to induce chondrogenesis with MEM alpha supplemented with 1% FBS, 6.25 μg/ml insulin, 50 μM ascorbic acid and 10 ng/ml transforming growth factor (TGF)-β1 (R&D Systems, USA) with medium changes every three days. Proteoglycan production was determined using toluidine blue O (Sigma Aldrich) staining. The proliferation ability of BMMSCs was determined using a Colony Formation Assay. The BMMSCs were seeded at a density of 200 cells/9.01 cm^2 culture dish. After incubation for 10 days, the colonies formed were fixed with methanol and stained with Giemsa solution (Sigma Aldrich). A cluster of at last 20 cells was defined as a colony-forming unit (CFU). CFU size and number were enumerated by microscope.

Osteogenic activity

To examine the osteogenesis activity of the cultured BMMSCs on the modified culture surfaces, characteristic ALP expression was examined at day 14, and calcium mineralization was examined at day 21. The studied culture surfaces were modified with PPX-ss-amine coating and the immobilization of BMP-2 protein. Subsequent detachment modification was performed using the time course at day 0, day 4, day 8 and day 12. A control experiment using the non-detachable PPX-amine coating with the same detachment treatment was conducted in parallel for comparison. Specifically, the cells were fixed with 4% paraformaldehyde for 30 min and then stained with an Alkaline Phosphatase Detection kit (Millipore, USA) in the dark for 30 min for ALP expression analysis. With respect to analyzing calcium mineralization, the fixed cells were stained with a 2% Alizarin red staining solution for 30 min, and the samples were thus used for further characterization. Three washing cycles were performed using deionized water during each staining process. Each experiment was conducted in triplicate.

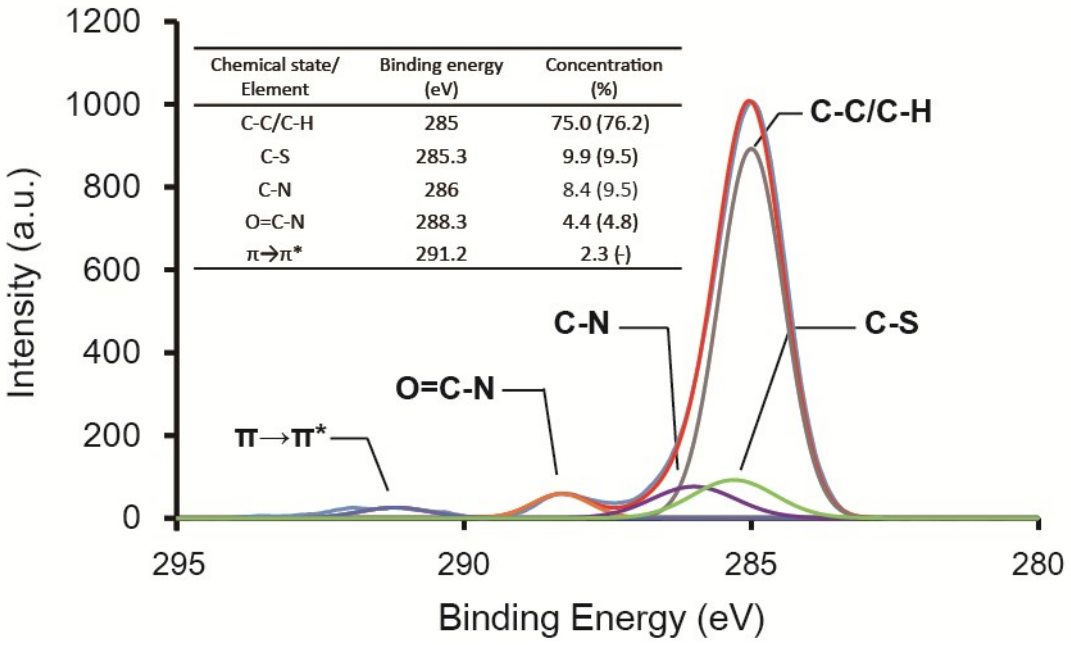

Figure S1. XPS high-resolution C_{1s} spectra of the PPX-ss-amine. The experimental results of the XPS survey high-resolution spectra are compared with the calculated values (in brackets) in the table. The signal at 285.0 eV is attributed to aliphatic and aromatic carbons (C–C, C–H), and the intensity at 75.0 atom% agrees well with the theoretical value of 76.2 atom%. The C–S bond was detected at 9.9 atom%, which agrees well with the theoretical value of 9.5 atom%. The C–N bond, which was detected at 8.4 atom%, approaches the theoretical value of 9.5 atom%. The peak at 288.3 eV is assigned to the O=C–N group, and the experimental value of 4.4 atom% agrees well with the theoretical value of 4.8 atom%. The signal at 291.2 eV (2.3 atom%) indicates π→π* transitions.

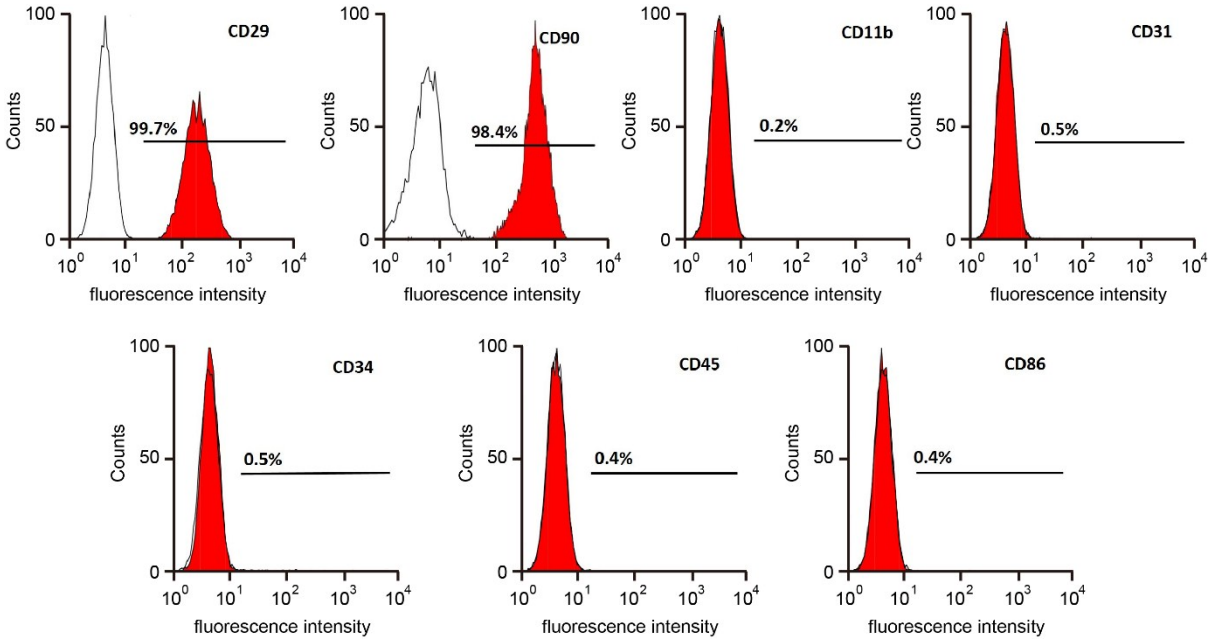

Figure S2. Immunophenotypes of BMMSCs were examined by using flow cytometry analysis. These cells were positive for CD29 and CD90, which represent adhesion cells and a general stem cell surface marker, respectively, and negative for leukocyte markers such as CD11b, CD31, CD34, and CD45, which represent endothelial cells, platelets and neutrophils, respectively, and were not expressed by these cells; neither was CD86, the costimulatory molecule.

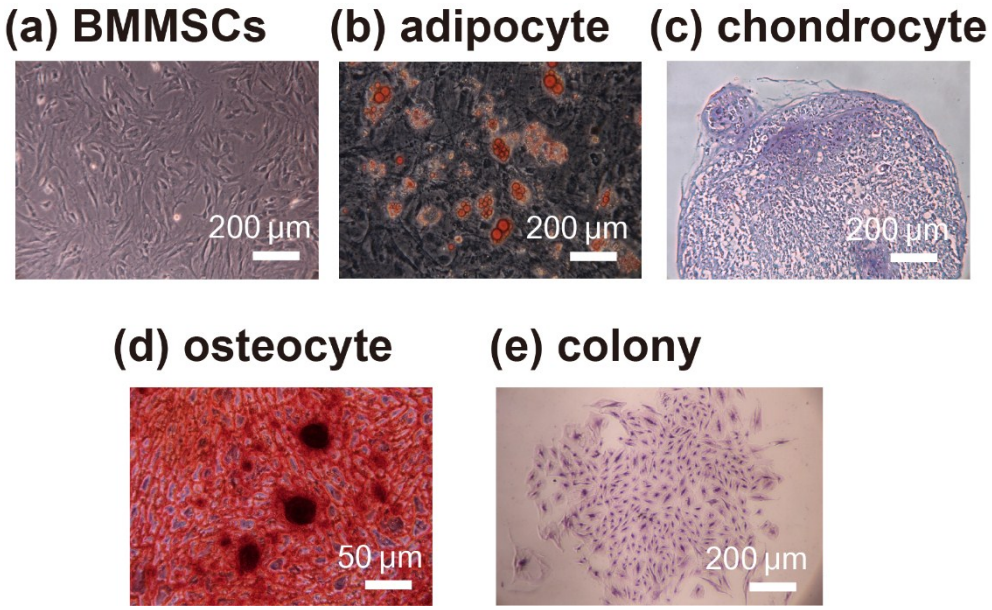

Figure S3. Differentiation and proliferation ability of BMMSCs were examined by culturing BMMSCs in different induction conditions. (a) Morphology of the BMMSCs was observed at day 4. (b) Adipocytes differentiated from the BMMSCs, and lipid droplets were stained by Oil Red O at day 21. (c) Chondrocytes differentiated from the BMMSCs, and proteoglycan production was stained by toluidine blue O at day 14. (d) Osteocytes differentiated from the BMMSCs, and calcium deposition was stained by alizarin red at day 14. (e) Colony formation of BMMSCs was stained by Giemsa after 10 days of culture.

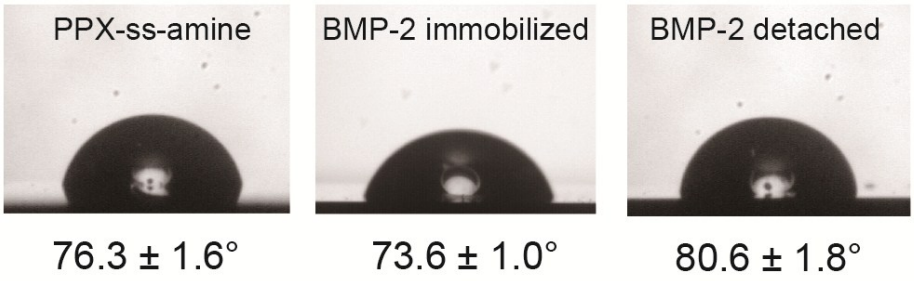

Figure S4. Surface wettability was confirmed by measuring the water contact angle of PPX-ss-amine coating surface, BMP-2 immobilized surface, and BMP-2 detached surface.

Reference

1. Z.-Y. Guan, C.-Y. Wu and H.-Y. Chen, *ACS Biomaterials Science & Engineering*, 2017, **3**, 1815-1821.